CHARACTIZATION OF

INTERGRASE INTERACTOR 1

BINDING CHROMATIN

By

DUSTIN JAMES STEELE

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Oklahoma State University

Stillwater, Oklahoma

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CHARACTIZATION OF

INTERGRASE INTERACTOR 1

BINDING CHROMATIN

Approved:

Donald Ruhl Ph.D.

Adviser

Patricia Canaan Ph.D.

Robert Matts Ph.D.

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I did my best.

Name: Dustin J Steele

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Abstract:

The remodeling of chromatin is a poorly understood and vital cellular process that plays a key role in an array of cellular functions including transcription, replication, and DNA damage repair. Studies have shown the importance of chromatin remodeling complexes like Human SWI/SNF in the development of diseases like cancer. Therefore, understanding the functions of the protein components of the complex is vital. Human INI1 is one such protein; INI1 is evolutionarily conserved and vital for proper SWI/SNF function. This study attempts to address's INI1's ability to interact with chromatin. Additionally, the determination that INI1 binding could demonstrate differential binding to linker DNA and the nucleosome, which make up chromatin, is addressed. The understanding INI1's binding is a crucial step in the understanding of SWI/SNF and the chromatin remodeling process.

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TABLE OF CONTENTS

Chapter Page

LIST OF ABBREVIATIONS

Abbreviation	Meaning
INI1	Integrase Interactor 1
SWI/SNF	Switching/Sucrose Non-Fermenting
DB	Dialysis Buffer
RB	Reaction Buffer
DBD	DNA Binding Domain
EMSA	Electro Mobility Shift Assay
Bp	Base pair
HIS tag	Histidine Affinity Tag
NPE	Nucleosome Positioning Element

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Regulation of DNA structure is a vital function of the eukaryotic cell. The packaging and unpacking of DNA into nucleosomes and higher order structures of chromatin as well as unstructured "naked" regions have a profound effect on the cellular functions and processes there within. These structures are an important aspect in several cellular processes including: DNA damage repair, transcriptional regulation, and cell cycle regulation. Due to the importance of these functions understanding how and what binds to the either the packed and/or unpacked DNA is vital to understanding how proteins play a role in the regulation of the processes listed above and what the impacts of that regulation is on downstream effects.

"Naked" DNA

Deoxyribonucleic Acid is the basic genetic information storage molecule. DNA is made of four bases: adenine, cytosine, guanine, and thymine. These four basic units pair together in preferential bonding pairs, the pairs are: adenine: thymine and cytosine: guanine. These base pairs are stacked upon one another and linked together into long a strand which twists upon its self. This twist results in a flexible double helix

DNA can take on multiple forms, the three most recognized are: A, B, Z. A-DNA is denoted by quite deep and narrow major groove and a very shallow and wide minor groove [42]. B-DNA is denoted by a shallow and wide major groove and a narrow and deep minor groove [41]. B-DNA is the general form DNA is thought to take in vivo, when not associated with other interactors, i.e. "naked". Z-DNA stands out as being the only left handed known form of DNA it also does not have a pounced major groove and a narrow and deep minor groove and it also shows a zig-zag pattern with the base pairs [40].

Mononucleosome

Chromatin structures makes up the majority of the genomic DNA landscape within the nucleus. It is a nucleoprotein complex that is conserved throughout the entirety of eukaryotes. As such, the remodeling of chromatin is an important component of many cellular processes due to the immobile nature of the core histones of the nucleosome. Chromatin comes in two forms heterochromatin and euchromatin. These are two widely different chromatin architectural forms. Heterochromatin is noted by loose nucleosome compaction and active transcription. While euchromatin, is known for tight nucleosome compaction as well as being noted for a distinctive lack of active transcription. Therefore the nucleosome is the basic subunit of these two vastly different forms of chromatin architecture. The mononucleosome thus represents the most basic unit of the chromatin. Mononucleosomes are defined by a flat left handed super helix of DNA which surrounds an octamer core of four histones in a heterotetramer of heterodimers. The histone core is made up of a tetramer of two H3 and H4 dimers and two dimers of H2A and H2B. The four core histones all contain a histone fold domain. The domain is characterized by three α-helices separated by two unstructured loops. This motif forms a quasi-two fold symmetry during dimer formation between H2A and H2B as well as H3 and H4 dimer pair [33]. The proteins are

held together through four-helix bundles, linking H3 to H3'and H2b to H4 [33]. The histone core is surrounded by 147 base pairs of DNA is structured in a super helix [7, 33].

Nucleosome Positioning Element and Nucleosome Assembly

The NPE is a segment of DNA that contains a region that will preferentially bind core histone octamer. The NPE were selected in vitro with a preference for high positioning power, i.e. the ability of the DNA to interact with a histone. These segments of DNA allow the assembly of nucleosomes into specific positions within the DNA segment. Additionally, equilibrium within the nucleosome segment will be obtained naturally [37]. Thus in vivo distances will be maintained during reassembly. The interaction of the nucleoprotein complex can be disrupted by salt concentration. Using an increasing salt concentration the histone octamer will break apart into its constituent parts, H2A/H2B dimer and H3/H4 tetramer. The complex disassociates in two molar NaCl. However, by lowering the salt concentration the nucleosome has the ability to reassembly [38, 39].

DNA Binding Domain

Protein DNA binding domains (DBD) can be divided in to groups around the secondary amino acid structure. DBD's that contain mostly alpha helical structures include: helix-turn-helix, helix-loop-helix, and leucine zippers [43]. The group is distinguished by an affinity for the major groove of DNA. Interaction is typically done through one of the helixes into a DNA groove (43). Proteins containing mostly beta strands are a second group they include: TATA box binding proteins, Immunoglobulin-like beta-sandwich, and beta-trefoil [40]. These proteins-DNA interactions are less common than the previous group. They also have more varied interactions owing to the structure of the beta strand and that loops between the strands provides the binding pattern [44-46]. The final group is an alpha helix and beta strand mix. This group includes: zinc

finger proteins and ribbon-helix-helix [40]. The leucine zipper and ribbon-helix-helix are also an example of a DBD that also has a dimerization domain [40]. The cooperative dimerization allows for greater discrepancy during an interaction. This discrepancy can be the further stabilization of other DBD on the same or other proteins. The alternative is that one binding event can lead to the destabilization of the same or other proteins interacting with the DNA [35]. The positive cooperative binding like in the zinc finger proteins or some of the above examples can lead to further protein binding events [40]. This ability allows for the assembly of multi-protein complexes on to a specific DNA location; thus allowing for a multitude of cellular processes.

DNA-Protein Interactions

The recognition of DNA by proteins is a vital aspect in cellular processes such as DNA damage repair, transcription, and replication and many others. The ways in which proteins identify DNA targets and attaches to the DNA does vary. These differences do have a common theme in that the proteins are adopting shapes that take on specific originations that promote specific and somewhat unique interactions.

There are a number of interactions that will occur between the protein and DNA due to the local and global topography that dictate specific or non-specific binding. Specific versus non-specific binding are terms that refer to the degree of affinity protein-DNA binding [40]. The difference between the specific and non-specific is the degree uniqueness of the binding patterns, as in the uniqueness or the universality of the pattern of hydrogen bonds, Van Der Waals forces, and other non-covalent bonds that contribute to the binding event. The two prominent contributors to direct interactions are hydrogen bonding and hydrophobic interaction [40]. The local structure of the DNA, hydrogen bonding or hydrophobic interaction can prevail as the specificity site for proteins [47-49]. These interactions in combination with the shape of the DNA

account for the ability of some protein to bind to diverse segments of DNA and other to have a "fine-tuned" specificity [50-51].

Nucleosome-Protein Interactions

Nucleosomes are a complex of DNA and protein. The very structure of nucleosomes leads to changes within the local topography of the DNA such that some proteins are no longer able to bind to DNA. An example is the TATA box binding protein which cannot bind DNA wrapped around the core histones because the DNA minor groove is no longer in an orientation that is capable of recognition and thus binding to the protein [52]. The nucleosome is also associated with a number of modifications to both the DNA and the histones. The most notable DNA modification is the methylation of cytosine residues. There are a number of histone modifications that impact the structure of the nucleosome. These modifications include: methylation, phosphorylation, acetylation, crotonylation, butyrylation, and ubiquination [34, 53]. The histone post translational modifications (PTM) are typically found on the histone tails. The PTMs listed are also examples of nucleosome-protein interaction. The orientation of the DNA around the histone also plays a role in the ways protein can target the nucleosome. The DNA is wrapped around the long axis of the histone octamer [33]. This orientation allows for interaction with the faces of the histone octamer. One aspect of this is the presence of an acidic patch on the H2A/H2B surface [33, 53]. Taken together the histone octamer and DNA provide two distinct targets for unique binding patterns. Nucleosomal recognition can thus be achieved by interaction with the histone octamer directly or DNA directly, recognition of either histones or DNA the cooperative binding to the other respectively, or recognition of histone tail/ tail PTM which induces further binding [34, 53].

SWI/SNF

Several protein complexes are involved in the remodeling of chromatin architecture including: SWI/SNF, NURF, NuRD, INO80, and ISWI [5]. SWI/SNF (mating type switch/sucrose non-fermenting) is well-studied and the first of the chromatin remodeling complexes to be investigated. The complex was first identified in *S. cerevisiae*. SWI/SNF is known to be highly conserved thought out all eukaryotic organisms [18]. SWI/SNF's ability to use the power of ATP hydrolysis to regulate transcription through changes in the chromatin architecture has been well noted. The complex performs this function by occluding or exposing the DNA sites required for binding of transcription factors, the basal transcription protein machinery and RNA polymerase II [29-31]. The SWI/SNF complex is a multi-subunit protein complex comprised of 9-14 subunits. The complex is comprised of a set of core proteins and peripheral proteins. Integrase interactor 1 (INI1; also known as SMRCAB1, SNF5, and BAF47) is known as one of the core subunits and is conserved in eukaryotes [1]. There is little known about the function of the non-enzymatic protein subunits. Understanding these proteins is essential to elucidating the function of SWI/SNF due to their presence within the core of the complex. INI1 is of particular interest due to its implication in human pathogenesis. Little is known about how INI1 interacts with nucleosome or linker DNA or where specific region of binding is.

INI1

Integrase Interactor 1 (INI1) was first described in as yeast-two hybrid assay. It was shown to interact with HIV-1 integrase [11]. INI1 is shown to be evolutionarily conserved because of this INI1 is also known as BAF47, (in *Drosophila*), SNF5 (in *Saccharomyces*, and SMARCB1 (in Human). INI1 is a protein of many roles. INI1 is shown to play a role in several

regulation complexes in addition to independent function and interactions that have implications throughout the cell; including cell cycle, cell differentiation, cytoskeleton organization, DNA damage repair, and viral functions [8-10, 15-17, 20-25]. There are two human isoforms of INI1. The two isoforms differ by nine amino acids in the 68aa-77aa. INI1 is a 47kDa protein that contains four known domains (Figure 1). The domains are a repeat 1 (Rpt1), repeat 2 (Rpt2), a putative coiled-coil domain (CCD), and putative DNA binding domain (DBD). All of the domains appear to be essential to function in higher eukaryotes [18, 19]. One of INI1's well known roles is as a tumor suppressor. This ability appears to be impaired if truncations exist at either the N-terminus or the C-terminus of the protein. Atypical Rhabdoid/Tatriod type tumors are rare and noted by disruptions/truncations within INI1 [26-28]. As such understanding the domains functions of INI1 are vital to its puzzle.

The Domains of INI1

The Rpt1 and Rpt2 domains are known protein-protein interactors. The two domains have conservation from all hierarchy of eukaryotes. These two domains are defined by the presence of two imperfect repeats of one another. These motifs have no homology to any known domains but they are essential for cellular and viral protein interactions [2, 3]. The Rpt's have been shown to be the predominate protein-protein interactors of INI1.

INI1 Domain Map

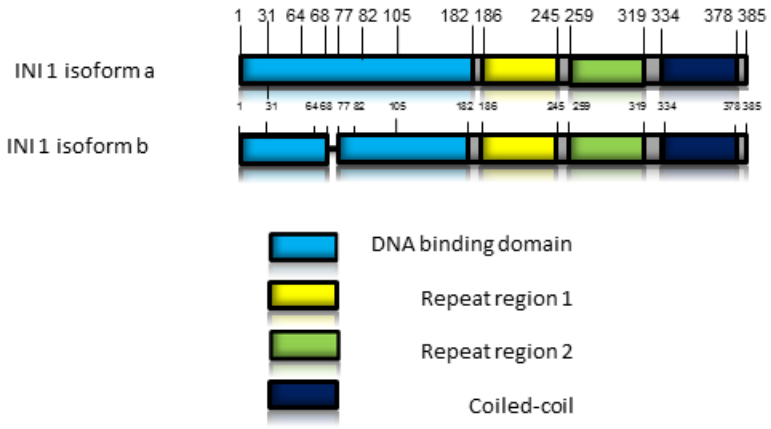

Fig1: Domain Map of INI1

It is through the Rpt region that INI1 is thought to interact with proteins like c-MYC and HIV integrase. Due to the uniqueness of the regions these interactions are important to understand to derive further investigations. Rpt2 is shown to have a NES (nuclear export sequence). The presence of the sequence is necessary for INI1 to complete several of its non-nuclear related functions e.g. shuttling of integrase and IFN signaling [13, 21]. INI1 has been shown to constantly travel between the nucleus and cytosol [14]. The cNLS Mapper program indicates the presence of a NLS (nuclear localization sequence) in the c-terminal of DBD [55-57]. The presence of the sequence provides further evidence for INI1 shuttling ability. The CCD is a putative protein-protein interaction domain [3, 4]. The CCD may play a role in the possible dimerization of INI1. It may also play a role in securing INI1 in the protein complex it associates with. The role of this domain within INI1 and within the SWI/SNF complex is not well understood. The DBD of INI1 is vital for many of the different functions and its role in the SWI complex. However, information is lacking about INI1's contribution to the stability of the nucleosome-chromatin-remodeler complex. This fact and the ability of INI1 to affect transcription and recruitment to DNA damage sites indicate a sever gap in knowledge in this area. The N-terminus of INI1 has been shown to fold into a Winged Helix-turn-helix DNA binding domain. This region was defined to 10aa-110aa [12]. The Winged helix is a structure made of a beta strand followed by a helix-turn-helix and in known to interact with the major groove [54]. DNA binding activity has also been suggested for the region 105aa-186aa [2]. The region was also shown to have a potential phosphorylation site [6]. This data lends itself to furthering the knowledge gap and presses the need for further investigation. An aspect of the DBD that has been thus far overlooked is the interaction with nucleosomes.

CHAPTER II

MATERIALS and METHODS

PCR

10X Thermopol reaction buffer (200 mM Tris base, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1.0% triton X-100), 10 mM dNTP's, TAQ polymerase 2 u/µl, 4µM forward primer, 4 µM reverse primer, 20 ng/µl Template DNA, 100 mM $MgCl_2$, 30 µM Dimethyl Sulfoxide

PCR was done on cDNA and 147 bp **N**ucleosome **P**ositioning **E**lement of 601 NPE [37]

147 Forward primer: 5'-CTGGAGAATCCCGGTGCC-3'

147 Reverse primer: 5'-ACAGGATGTATATATCTG-3'

Clean up of PCR product is done by phenol –chloroform clean up (Phenol added to equal volume of PCR then centrifuged followed by 1:1 phenol/chloroform added to equal volume of PCR then centrifuged followed by chloroform added to equal volume of PCR then centrifuged. The aqueous layer taken during each step) ethanol precipitation (95% ethanol at 2.5X volume, $1/10^{th}$ volume 3 M sodium acetate) placed in -80 degrees Celsius freezer overnight then centrifuged. The pellet is taken.

Monoucleosome Assembly

HeLa cell extracted core histones (H2A, H2B, H3, H4 data not shown) are added to with 500 ng/µl 147 bp DNA and 2X Assembly buffer (3.5 M NaCl, .05 M tris pH 7.5, 0.003 M EDTA, .03 DTT, .3 mg/ml BSA) into the cap of a siliconized microcentrifuge tube with a half of a dialysis tube stretched across with molecular weight cutoff of 3500 daltons secured the collar cut to allow dialysis. The chamber is placed into high salt buffer (2 M NaCl, .01 Tris pH 7.5, 100 mM EDTA, 0.2 g/ l sodium azide) then low salt buffer (0.25 M NaCl, .01 Tris pH 7.5, 100mM EDTA, 0.2 g/ l sodium azide) at 0.67 ml/min for 22 hrs then switch to 1.27 ml/min for 5 hrs. The chamber is then placed into zero salt buffer (0.01 Tris pH 7.5, 100 mM EDTA, 0.2 g /l sodium azide)

Mononucleosome Quantification

Mononucleosomes are run on a 6% native Page gel (0.05% glycerol, .25X TBE, 6% acrylamide mix-29:1 acrylamide:bis, 0.1% ammonium persulfate, TEMED) along with a four point titration of 147 bp DNA at different concentrations. Gel was post stained for 20 min with 10 mg/ml ethidium bromide (EtBr). Mononucleosomes are quantified by densitometry.

DNA Quantification

DNA was put on the Nanodrop for quantification which was read at 260 nm/280 nm

Cloning

PCR is performed on Human INI1 isoform B cDNA with HIS tag. cDNA PCR is cleaned by phenol/chloroform-ethanol precipitation. cDNA PCR is restriction digested (BamH1 and Nde1) and ligated into an inducible plasmid. Plasmid is transformed into *E. coli* and isolated by kanamycin selection. E. Coli containing protein expression plasmid are grown in 1/5 volume ratio in a two liter Erlenmeyer flask in Luria broth (1% tryptone, 0.5% yeast extract, 1% NaCl, 10 mg/ml kanamycin).

Protein Purification

Ni-NTA resin is resuspended and wash with equilibration buffer pH 7.5 (10 mM Tris pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 10% glycerol, 0.1% NP 40 (IGEPAL)). Cultures, grown to 600 nm optical density, containing protein of interest are pelleted and then resuspension in lysis buffer (25 mM Tris pH 7.5, 0.5 M NaCl, 0.5 mM EDTA, 10% glycerol, 10 mM imidazole, 1% NP40 (IGEPAL) 0.1% SDS). The solution is then sonicated for 2:00 mins for 10 sec pulses. The lysate is centrifuged and the supernatant is collected. The cell extract is incubated with the Ni-NTA resin for 2-10 hrs. The cell extract and resin are gravity packed into a 3cm column with a bed of glass wool. Wash buffer (10 mM tris pH7.5, 0.5 M NaCl, 10% glycerol, 15 mM imidazole, 0.2% NP40 (IGEPAL)) is then run over the column overnight. Run elution buffer pH 7.5 (10 mM Tris pH 7.5, 200 mM NaCl, 10% glycerol, 250 mM imidazole, 0.1% NP40) over the column and collect 1 ml fractions into 1.5 ml microcentrifuge. The proteins were visualized be staining with GelCode Blue safe protein stain-Thermo

Protein Dialysis

Protein fractions are dialyzed DB buffer (20 mM Tris pH 7.5, 10% glycerol, 0.2 mM EDTA, 100 mM NaCl, add fresh 0.2 mM PMSF, 1.0 mM DTT) in a 200 ml DB:1 ml of sample overnight.

Protein Quantification

Protein fraction were run on 12%-18% Polyacrylamide SDS gels for 70 mins at 120 v (30% acrylamide mix-29:1 acrylamide:bis, 380 mM tris pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, TEMED) with a four point titration of 0.25 µg/µl BSA. 5 µl of loading buffer (Laemmli Sample Buffer from BIO-RAD) was added to all samples. Samples were boiled for 5 mins. Then run on SDS-PAGE. Gels were washed after run three times in H_2O then visualized by gel co-blue stain overnight. Proteins were then quantified by densitometry.

Electro Mobility Shift Assay-147 bp DNA

The protein sample along with 147 bp DNA, DB buffer, and DNA reaction buffer (RB) (glycerol 46%, 0.7 mM EDTA, 0.12 M HEPES, 0.028 M DTT, 0.03 M $MgCl_2$, 0.1 mg/ml BSA) was incubation at room temperature for 25 mins after after which 6 µl of 50% glycerol was added. The samples were incubated for 25 min., then were loaded onto a 6% native page gel and run for 75-90 min at 4^oC at 120 v. Gel is visualized by incubation with 10 mg/ml EtBr for 20 mins. All experiments were performed in a multiple replication.

Electro Mobility Shift Assay-Mononucleosome

The protein sample along with mononucleosome, DB buffer, and DNA reaction buffer (glycerol 46%, 0.4 M Tris pH7.5, 0.12 M HEPES, 0.028 M DTT, 0.030 M $MgCl_2$, 0.1 mg/ml BSA) was incubated for 25 mins at room temperature after which 6 µl of 50% glycerol was added. The samples were incubated for 25 min., then were loaded onto a 6% native page gel and run for 75-90 min at 4^oC at 120 v. Gel is visualized by incubation with 10 mg/ml EtBr for 20 mins. All experiments were performed in a multiple replication.

CHAPTER III

RESULTS

Mononucleosomes

The chromatin (10 nm fiber) can be divided into two groups. These groups are linker DNA and the nucleosome. the linker DNA is "naked" in that is not interaction with any proteins. The nucleosome consists of the histone core and 147 base pairs (bp) of DNA. "Linker DNA" is defined as the region of DNA between two nucleosomes. This region of DNA is not associated with the core histones (H2A, H2B, H3, H4) in the "beads on a string" structure of chromatin.

The 147 bp of 601 NPE segment amplified in PCR is the portion that serves as a preferential binding target site for the core histones in nucleosome assembly [37]. To assembly the mononucleosomes a reverse salt gradient was implemented. This was done by placing the 147 bp DNA in a dialysis chamber that contained the core histones (H2A, H2B, H3, H4) and assembly buffer. Salt concentration was titrated down from 2 M to a minimal of 0.6 M NaCl. This in vitro method allows the core histones the assembly on the positioning element in a similar way to the in vivo process.

The quantification of the mononucleosome was done by using a four point curve of 147 bp DNA on a native PAGE gel to compare to assembled mononucleosomes (Figure 2). The comparison was performed by a densitometry program.

Cloning and Purification INI1 and Truncations

Human INI1 truncations were selected based on attempt to determine the minimal region for function. After vector construction transformation into *E. coli* and culturing was done. Cultures were collected and pelleted. The expressed INI1 proteins are extracted by resuspending the cell culture pellet in lysis buffer. The resuspension was sonicated for two minutes in ten second intervals. This was followed by the separation of the "cellular debris" suspended proteins. The supernatant was incubated with NTA-Ni resin. To elute the INI1 clone imidazole was used to elute (Figure 3). Fractions were taken: these fractions were run on a SDS PAGE gel. Fractions containing the INI1 clone of interest in visual concentrations were further dialyzed in Dialysis Buffer (DB). This was done in part to significantly reduce the concentration of imidazole within the INI1 samples. The purified protein is run on a SDS PAGE gel along with a four point BSA standard curve. The densitometry program mentioned earlier is used to quantitate the given INI1 clone. The μM concentration is calculated to obtain a concentration useable in the EMSA.

Quantification of Mononucleosome

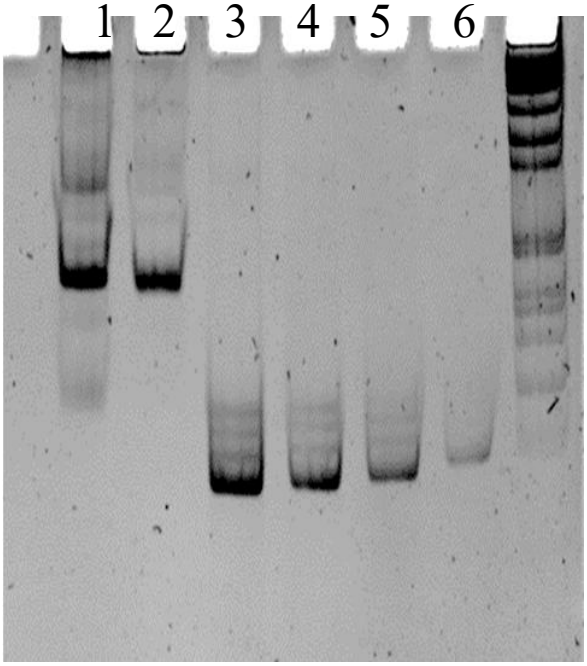

Fig2: 6% native PAGE. Lane 1) Mononucleosome sample 1. Lane 2) Mononucleosome sample 2. Lane 3) 750 ng/µL. Lane 4) 500 ng/µL. Lane 5) 250 ng/µL. Lane 6) 125 ng/µL.

Protein Purification of INI1 and Clones

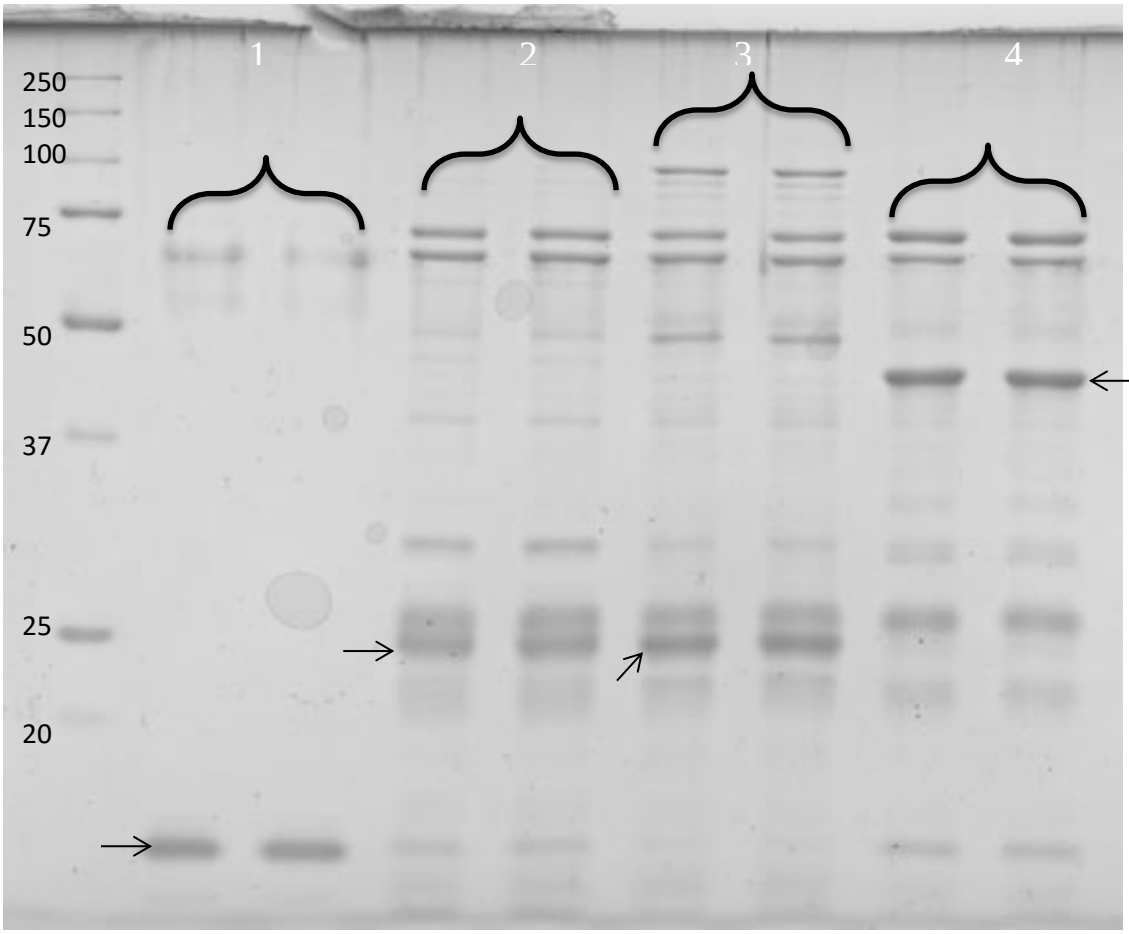

Fig3: 15% SDS PAGE. Run for70 min. at room temp. Lane 1) Δ1-104/Δ186-385 Lane 2) Δ186-385 Lane 3) Δ1-186 Lane 4) Full Length INI1

INI1 Δ186-385 Can Bind "Naked" DNA

Previous studies have provided evidence that INI1 has the ability to bind "naked" DNA [2, 12]. These studies came to different conclusions regarding the region that is binding to the "naked" DNA. Because of this difference existed the lab chose to begin the DNA binding domain (DBD) determination from square one. This involved testing to determine full length INI1's binding profile in an EMSA. An Electro Mobility Shift Assay (EMSA) allows the determination of a protein-DNA binding event by allowing a comparison between a control band and experimental band. In which the experimental band will have differential migration within the native PAGE when comparing to the progression of the control band.

To start the investigation "naked" DNA was looked at initially. The 147 bp of 601 NPE, that was amplified using PCR, was incubated at a single concentration with full length INI1 in a titration of increasing protein concentration from a ratio of 0.25:1 to 2:1 (protein: "Naked" DNA) also containing RB, DB and sterile water. The native PAGE gel showed full length INI1 did shift the "naked" DNA (Figure 4). This is consistent with previous studies [3]. Having confirmed that full length INI1 can bind "naked" DNA the lab used the previous studies to divide the protein in to two halves INI1 Δ186-385 and INI1 Δ1-186. These INI1 truncations were run separately in EMSAs. INI1 Δ186-385 was run in an EMSA in the same ratios as above, 0.25:1 to 2:1.

Titration of INI1 with "Naked" DNA

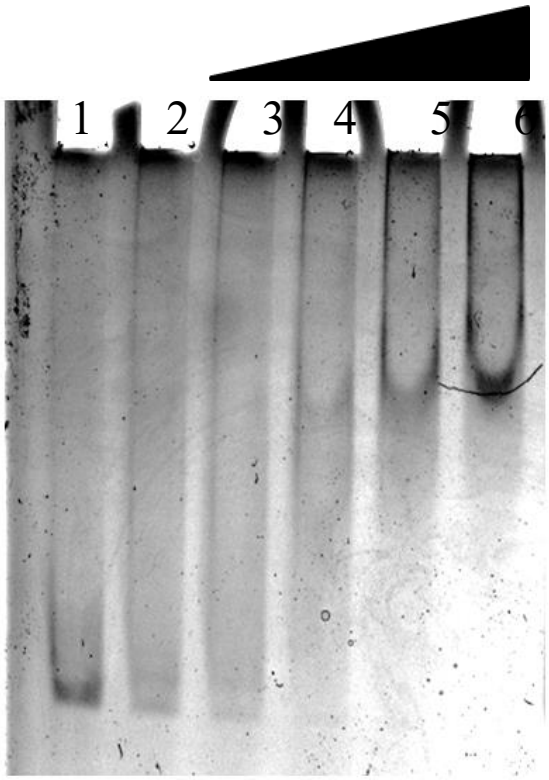

Fig4: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 2-6: full length INI1Ratio protein: "Naked" DNA: 1.)0:1 (0nM:150nM) 2.).25:1 (37.5nM:150nM) 3.).5:1 (75nM:150nM) 4.).75:1 (112.5nM:150nM) 5.)1:1 (150nM:150nM) 6.)2:1 (300nM:150nM)
Representative gel of n=3

The native gel showed that the truncation INI1 Δ186-385 was able to shift "naked" DNA (Figure 5). This was consistent with the previous studies as it encompasses both proposed DBDs. Next, INI1 Δ1-186 was run with "naked" DNA in an EMSA in the same manner as above (0.25:1 to 2:1). The native gel showed that truncation INI1 Δ1-186 was unable to shift "naked" DNA (Figure 6). The inability of Δ186-385 to bind is consistent with the current literature.

INI1 Δ186-385 Can Bind Mononucleosome

Despite the importance in SWI/SNF there is a lack of information on INI1's ability to interact with mononucleosomes. The lab began by testing full length INI1 for binding potential to mononucleosome. Full length Human INI1 was run in an EMSA by, incubation differing concentrations of protein with a single concentration of mononucleosomes in a ratio 0.25:1 to 2:1 (protein:Mononucleosome) also containing RB, DB and sterile water. The resulting native PAGE gel showed full length INI1 was able shift the mononucleosome (Figure 7). Next the lab tested INI1 Δ186-385 in an EMSA in a manner as stated above (0.25:1 to 2:1). The native gel showed that INI1 Δ186-385 was able to shift mononucleosome (Figure 8). INI1 Δ1-186 was the next truncation run in an EMSA. The EMSA was run a manner as above (0.25:1 to 2:1). The native gel indicated that INI1 Δ1-186 was unable to bind to mononucleosomes (Figure 8).

Determining Binding INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 to "Naked" DNA

Division of the INI1 Δ186-385 was done as previous studies had indicated that either truncation INI1 Δ1-104/Δ186-385 or INI1 Δ105-385 had the potential to bind DNA [12,2].

Titration of Δ186-385 with "Naked" DNA

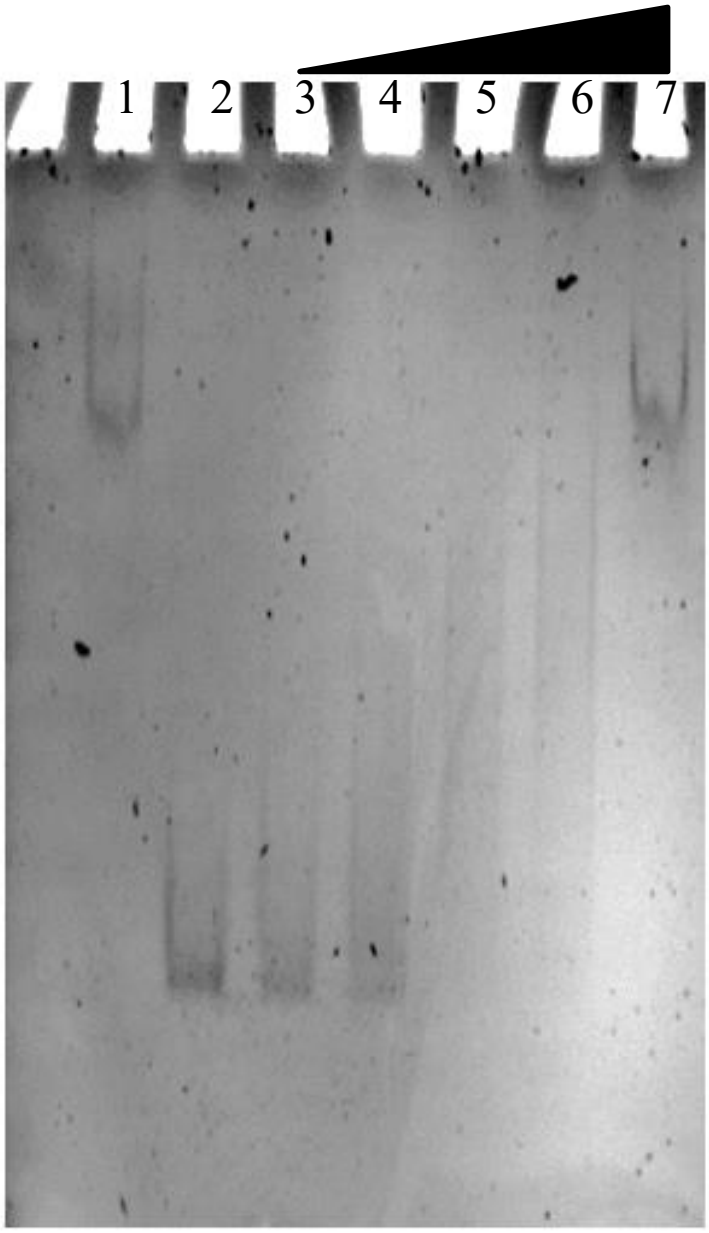

Fig 5: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ186-385. Ratio: protein:"Naked" DNA : 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM))0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM)
Representative gel of n=3

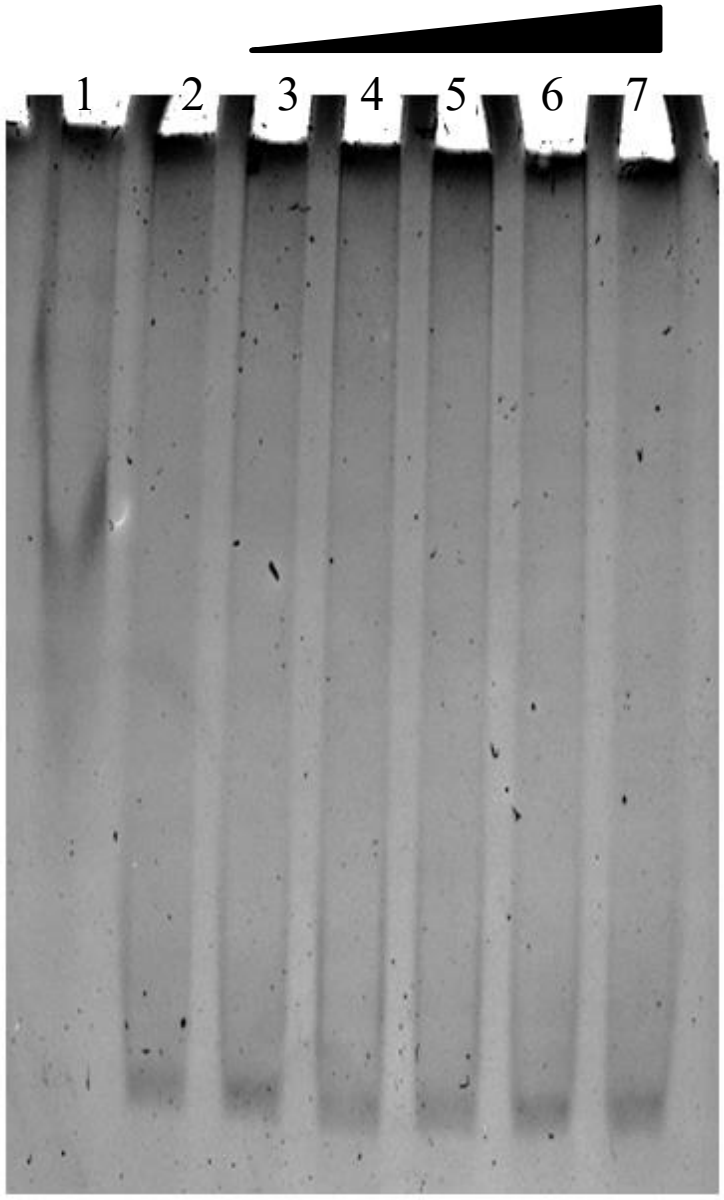

Fig6: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ1-186. Ratio: protein:"naked" DNA: 1) 2:1 (300nM:150nM 2)0:1 (0nM:150nM 3)0.25:1 (37.5nM:150nM) 4)0.5:1 (75nM:150nM) 5)0.75:1 (112.5nM:150nM) 6)1:1 (150nM:150nM) 7)2:1 (300nM:150nM)
Representative gel of n=3

Titration of INI1 with Mononucleosome

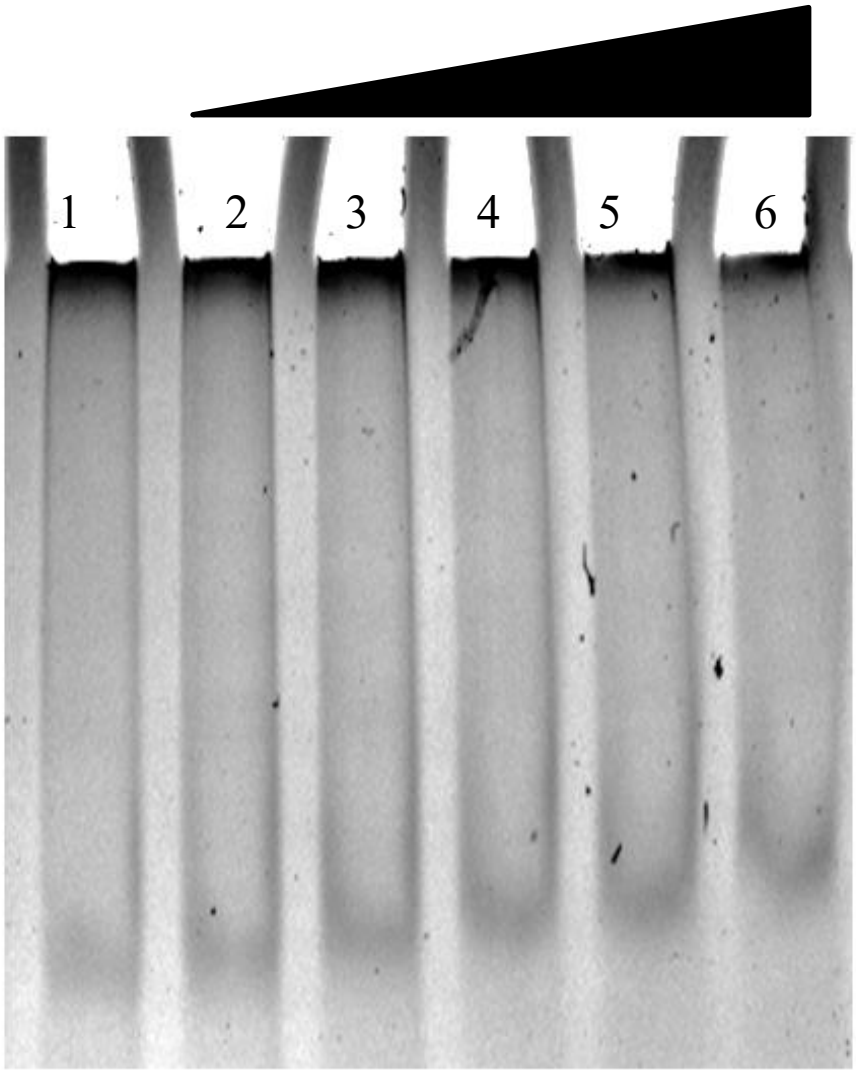

Fig7: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 2-6: full length INI1Ratio protein: Mononucleosome: 1)0:1 (0 nM:150 nM) 2)0.25:1 (37.5 nM:150 nM) 3)0.5:1 (75 nM:150 nM) 4)0.75:1 (112.5 nM:150 nM) 5)1:1 (150 nM:150 nM) 6)2:1 (300 nM:150 nM) Representative gel of n=3

Titration of Δ186-385 and Δ1-186 with Mononucleosome

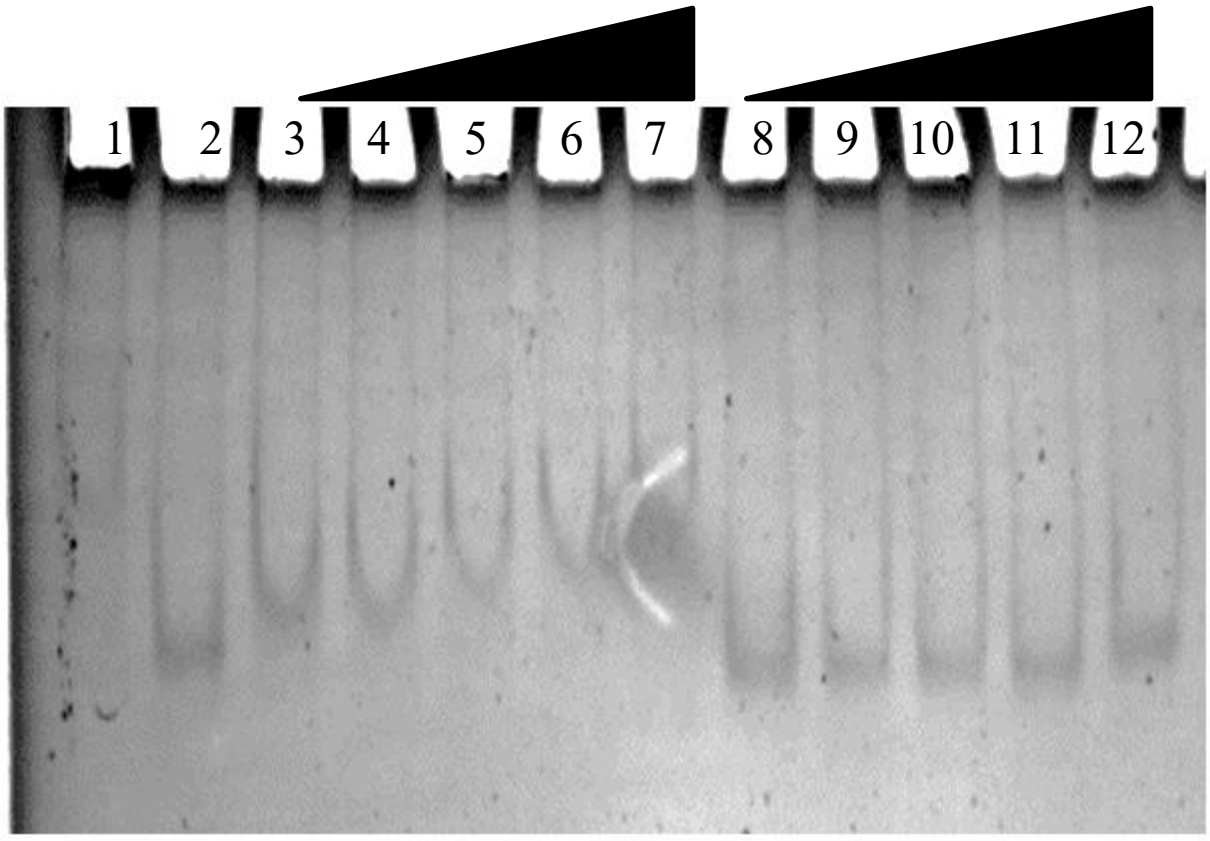

Fig8: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ186-385. Lane 8-12: Δ1-186. Ratio: protein:Mononucleosome: 1) 2:1 (300nM:150nM 2)0:1 (0nM:150nM 3)0.25:1 (37.5nM:150nM) 4)0.5:1 (75nM:150nM) 5)0.75:1 (112.5nM:150nM) 6)1:1 (150nM:150nM) 7)2:1 (300nM:150nM) 150nM 8)0.25:1 (37.5nM:150nM) 9)0.5:1 (75nM:150nM) 10)0.75:1 (112.5nM:150nM) 11)1:1 (150nM:150nM) 12)2:1 (300nM:150nM) Representative gel of n=3

The lab ran INI1 Δ1-104/Δ186-385 in an EMSA with a single "naked" DNA concentration and a titration of protein in a ratio of 0.25:1 to 2:1 (protein: "naked" DNA) also containing RB, DB and sterile water. The native PAGE gel showed no shifting of the "naked" DNA (Figure 9). INI1 Δ105-385 was next tested using an EMSA in a manner as above (0.25:1 to 2:1). The native gel indicated that no shift of the "naked" DNA (fig10).

Determining Binding INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 to Mononucleosome

Because, INI1 Δ186-385 was shown to interact with a mononucleosome and INI1 Δ1-104/Δ186-385 or INI1 Δ105-385 had the potential to bind DNA based on two previous studies [2,12].Showing the mononucleosomes binding ability of INI1 Δ186-385 the decision to divide the region in to the INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 was taken. An EMSA was run with a single mononucleosome concentration and a titration INI1 Δ1-104/Δ186-385 of in a ratio of 0.25:1 to 2:1 (protein:mononucleosome) also containing RB, DB and sterile water. The native PAGE gel showed that INI1 Δ1-104/Δ186-385 truncation was unable to shift the mononucleosome (Figure 11). The lab then proceeded to test INI1 Δ105-385 using an in a manner as above (0.25:1 to 2:1). The resulting native gel showed no shift in the mononucleosome (Figure 12).

Determining Binding INI1 Δ1-82/Δ186-385 and INI1 Δ1-62/Δ186-385 to "Naked" DNA

As Δ186-385 was able to shift "naked" DNA and INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 were not, the lab chose to determine how of Δ186-385 could be removed and still retain shifting ability.

Titration Δ1-104/Δ186-385 with "Naked" DNA

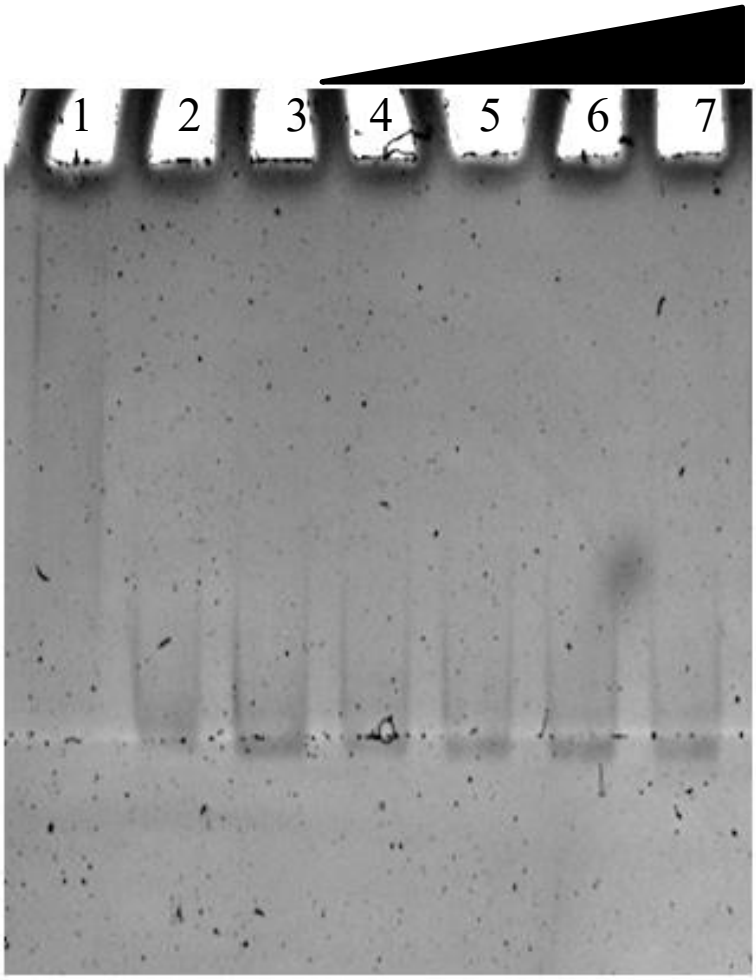

Fig9: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ1-104/Δ186-385. Ratio: protein:"naked" DNA: 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM) 4)0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM)
Representative gel of n=3

Titration of Δ105-385 with "Naked" DNA

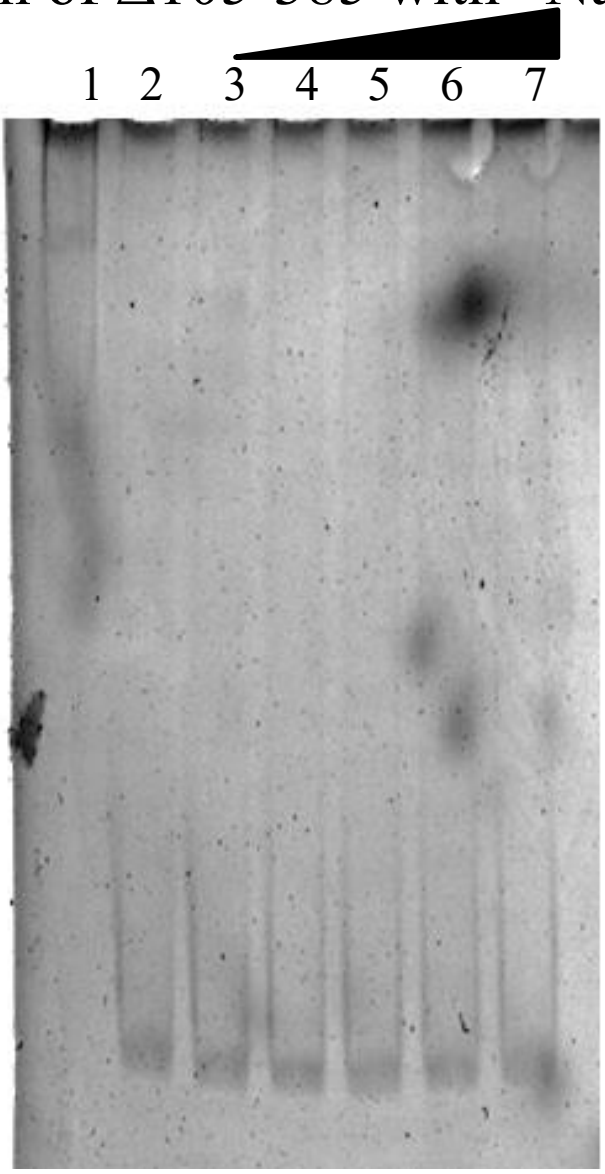

Fig10: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ105-385. Ratio: protein:"naked" DNA: 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM) 4)0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM)
Representative gel of n=3

Titration of Δ1-104/Δ186-385 with Mononucleosome

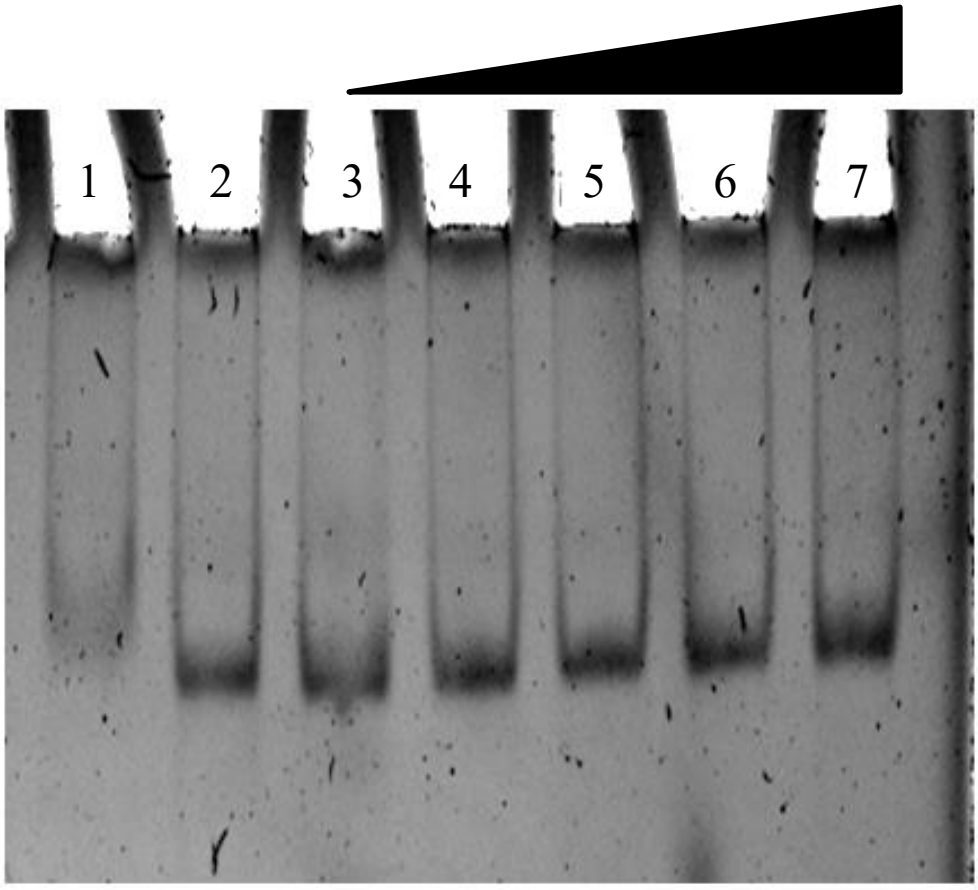

Fig11: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ1-104/Δ186-385. Ratio: protein: Mononucleosome : 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM) 4)0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM)
Representative gel of n=3

Titration of Δ105-385 with Mononucleosome

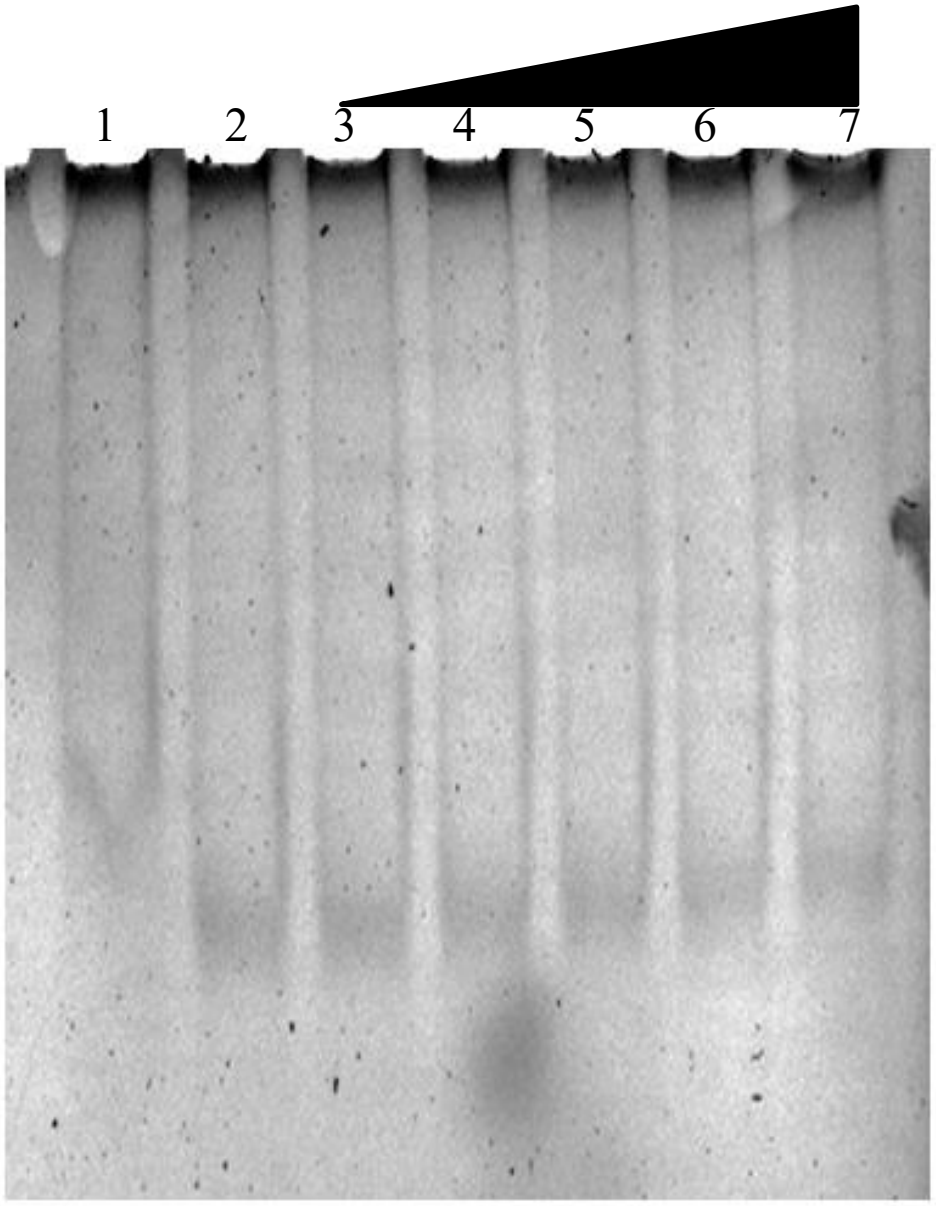

Fig12: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ105-385. Ratio: protein: Mononucleosome : 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM) 4)0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM)
Representative gel of n=3

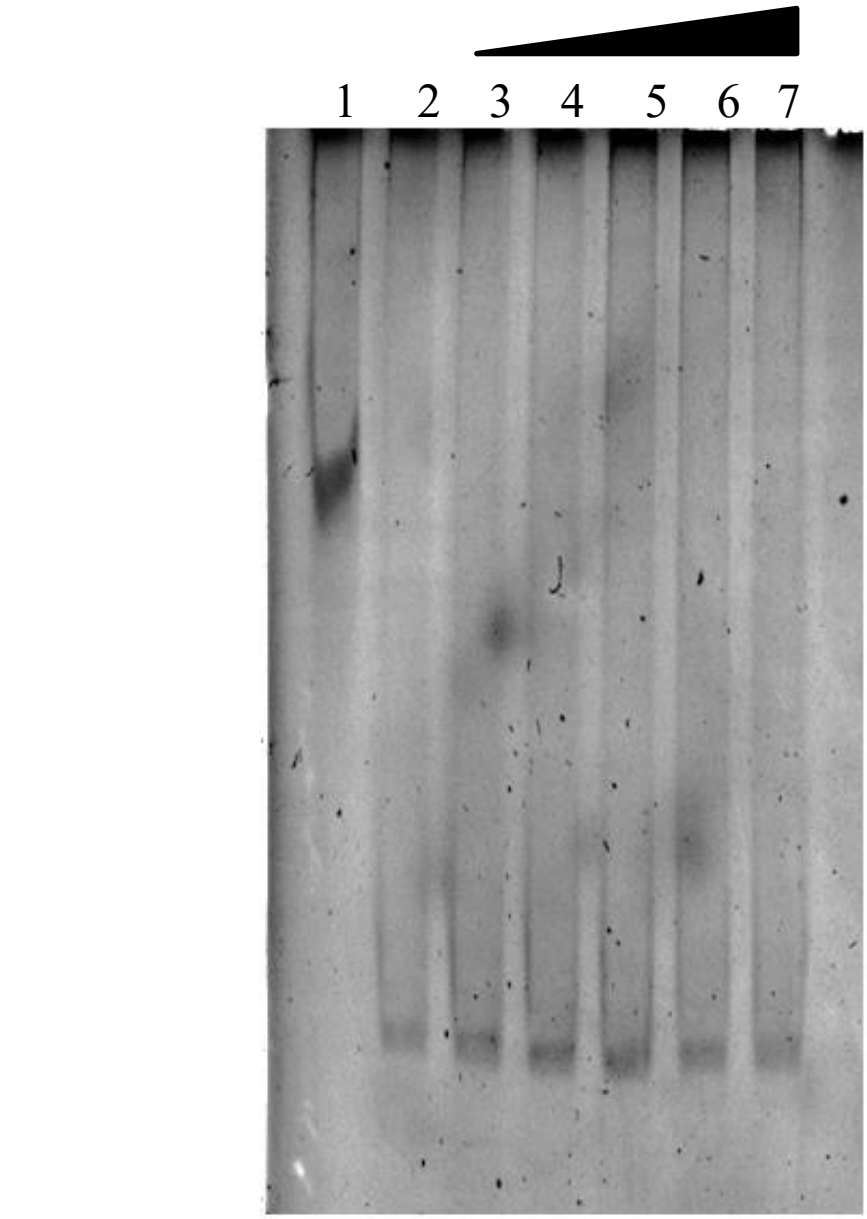

Fig13: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ1-81/Δ186-385. Ratio: protein: "Naked" DNA : 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM) 4)0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM) Representative gel of n=3

INI1 Δ1-82/Δ186-385 was run in an EMSA with a single "naked" DNA concentration and a titration of increasing protein concentration from a ratio of 0.25:1 to 2:1 (protein:"Naked" DNA) also containing RB, DB and sterile water. The native PAGE gel showed no shift of the "naked" DNA (Figure 13). After that the lab proceed to run an EMSA in a manner as above (0.25:1 to 2:1) with INI1 Δ1-62/Δ186-385. The native gel showed no shift with "naked" DNA (Figure 14).

Determining Binding INI1 Δ1-82/Δ186-385 and INI1 Δ1-62/Δ186-385 to Mononucleosome

The lab reasoned that because INI1 Δ186-385 had been shown to bind mononucleosome then systematic reduction of this region would be the best way to proceed to determining the minimal binding region of INI1. As such truncations INI1 Δ1-82/Δ186-385 and INI1 Δ1-62/Δ186-385 were made. First, the lab tested INI1 Δ1-82/Δ186-385 in an EMSA was run with a single mononucleosome concentration and a titration INI1 Δ1-82/Δ186-385 of in a ratio of 0.25:1 to 2:1 (protein:mononucleosome) also containing RB, DB and sterile water. The native PAGE gel indicated no shift of the mononucleosomes (Figure 15). Following this the Δ1-62/Δ186-385 was run in an EMSA in a manner as above. The native PAGE gel showed no shift of the mononucleosome (Figure 16).

Excess Protein Does Not Induce Shift

The lab decided to test high protein concentration within the EMSA. The three INI1 truncations Δ186-385, Δ1-104/Δ186-385, and Δ105-385 were run in an EMSA at two concentration points, 2:1 and 8.67:1 (protein:"Naked" DNA), also containing RB, DB and sterile water (Figure 17).

Titration of Δ1-63/Δ186-385 with "Naked" DNA

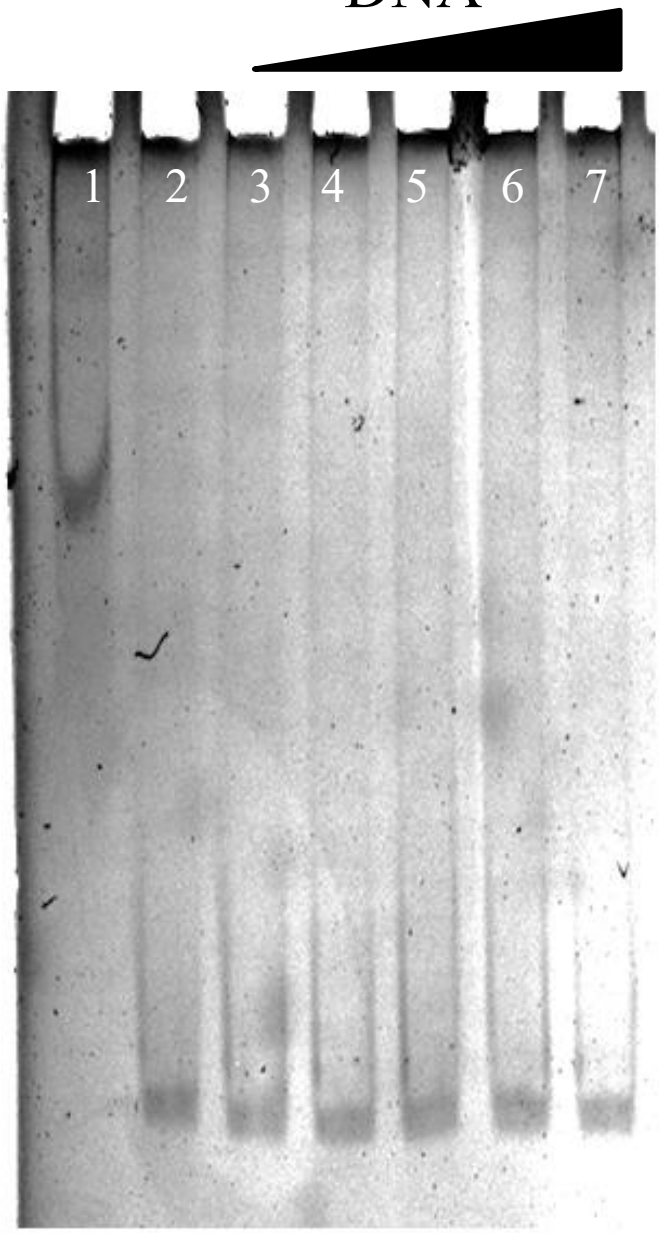

Fig14: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ1-63/Δ186-385. Ratio: protein: "Naked" DNA : 1) 2:1 (300 nM:150 nM) 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM) 4)0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM)
Representative gel of n=3

Titration of Δ1-81/Δ186-385 with Mononucleosome

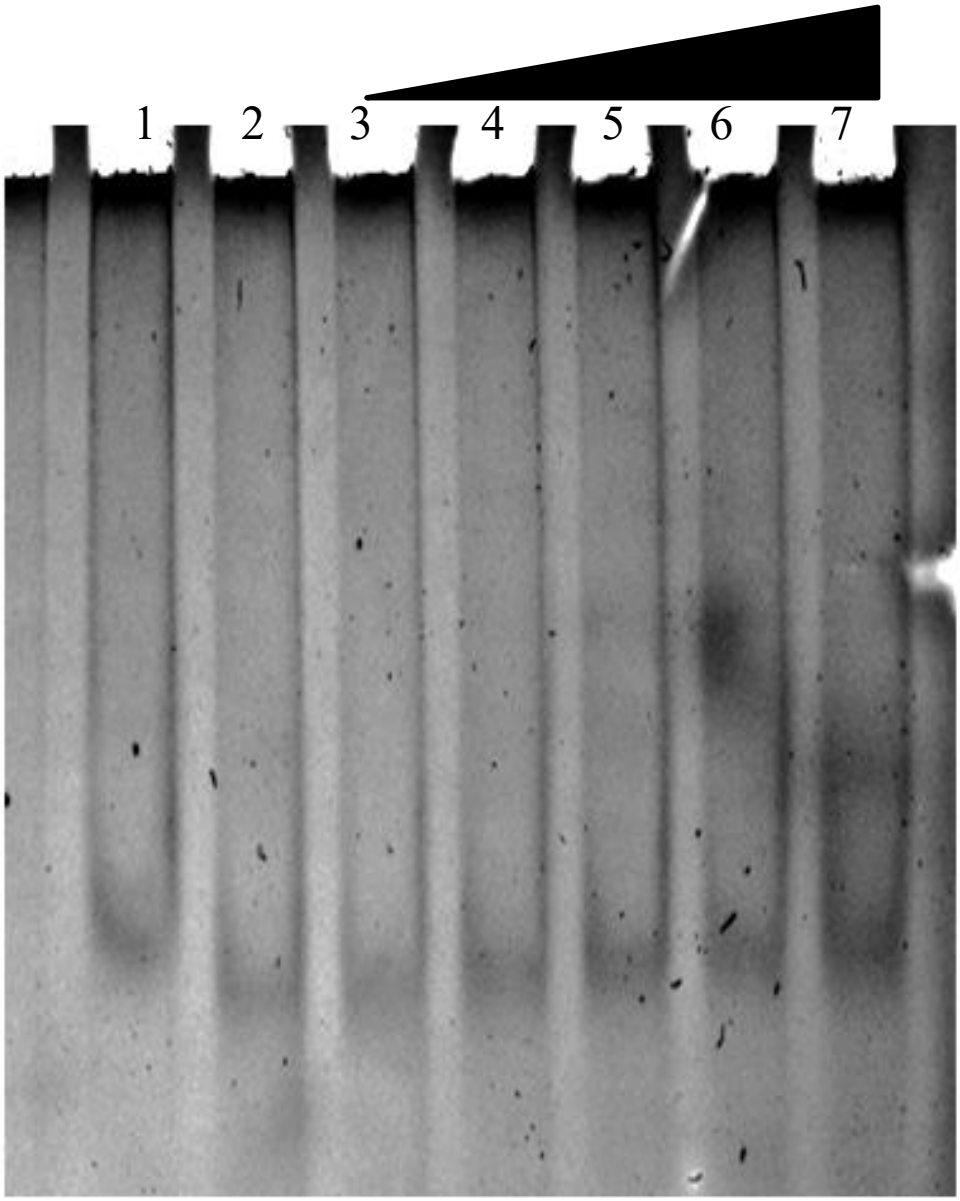

Fig15: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ1-81/Δ186-385. Ratio: protein: "Naked" DNA : 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3)0.25:1 (37.5 nM:150 nM) 4)0.5:1 (75 nM:150 nM) 5)0.75:1 (112.5 nM:150 nM) 6) 1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM) Representative gel of n=3

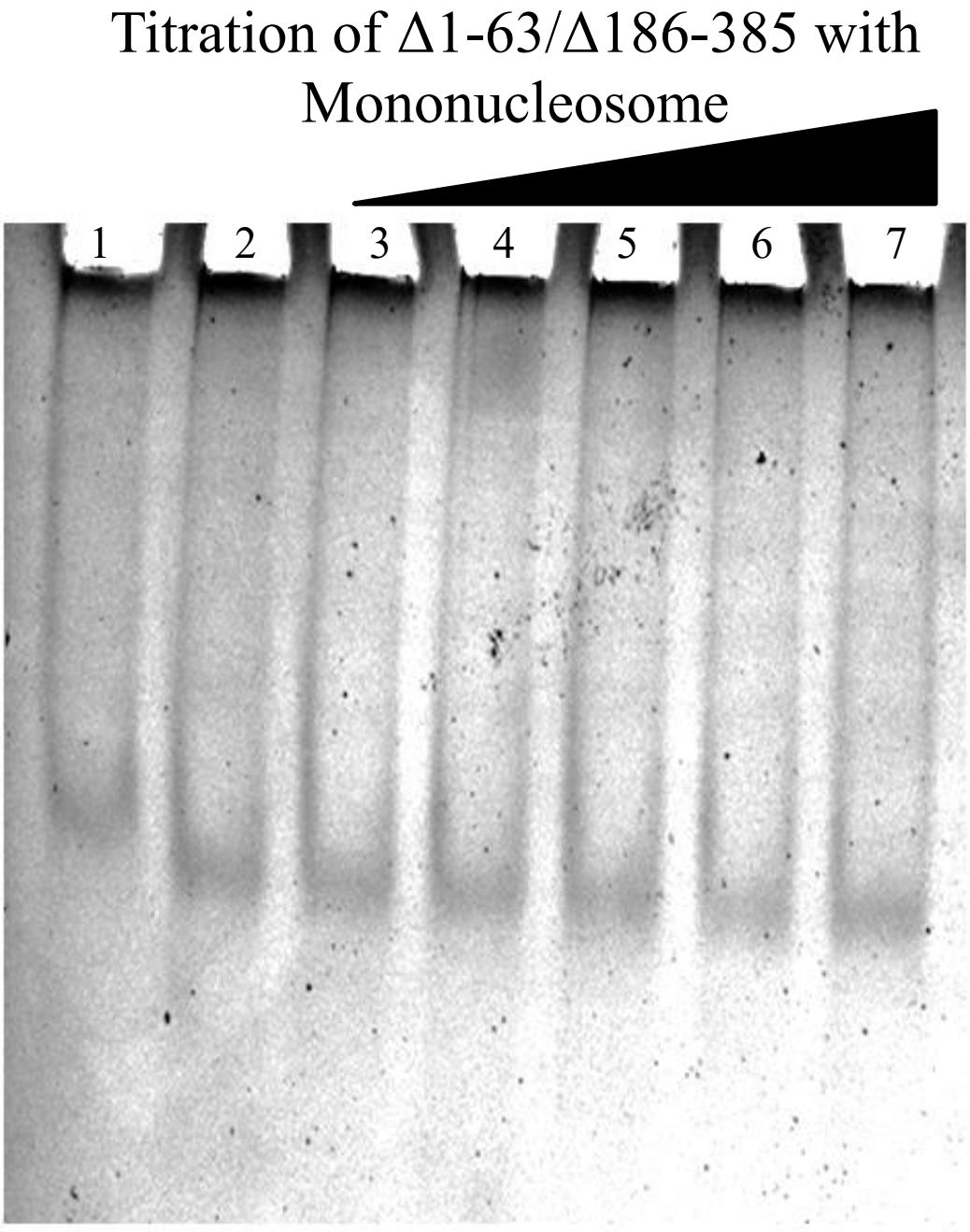

Fig16: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-7: Δ1-63/Δ186-385. Ratio: protein: Mononucleosome : 1) 2:1 (300 nM:150 nM 2)0:1 (0 nM:150 nM 3).25:1 (37.5 nM:150 nM) 4).5:1 (75 nM:150 nM) 5).75:1 (112.5 nM:150 nM) 6)1:1 (150 nM:150 nM) 7)2:1 (300 nM:150 nM) Representative gel of n=3

Titration of Δ1-186, Δ105-385, and Δ1-104/Δ186-385 at High Concentration

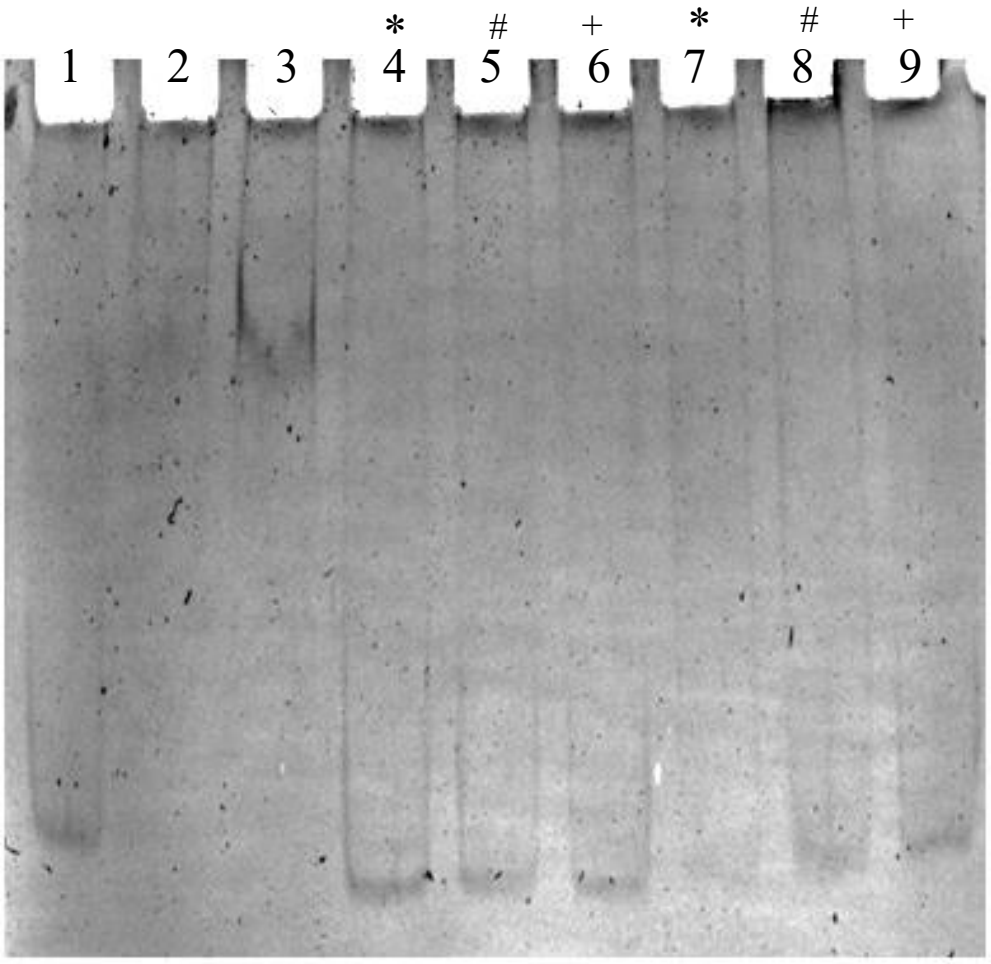

Fig17: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: no protein. Lane 2: full length INI1. Lane 3: Δ186-385. Lane 4,7: Δ1-186. Lane 5,8: Δ105-385. Lane 6, 9: Δ1-104Δ186-385. Ratio: protein:"Naked" DNA: 1)0:1 (0 nM:150 nM) 2) 2:1 (300nM:150nM) 3) 2:1 (300 nM:150 nM) 4)2:1 (300 nM:150 nM) 5) 2:1 (300 nM:150 nM) 6) 2:1 (300 nM:150 nM) 7)8.67:1 (1300 nM:150 nM) 8) 8.67:1 (1300 nM:150 nM) 9) 8.67:1 (1300 nM:150 nM)
Representative gel of n2

Intact Δ186-385 Required for Binding to "Naked" DNA and Mononucleosome

The lab added INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 in combination. The INI1 truncations were added in equal amounts of protein. The INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 combined samples were run in a three point titration. The three points are 0.5:1, 1:1, 2:1 (protein:"naked" DNA/mononucleosome) also containing RB, DB and sterile water... The native PAGE gel showed that the combined samples were not able to shift both "naked" DNA and mononucleosome (Figure 18, 19).

The lab added INI1 Δ1-62/Δ186-385 and INI1 Δ105-385 in combination. The INI1 truncations were added in equal amounts of protein. The INI1 Δ1-62/Δ186-385 and INI1 Δ105-385combined samples were run in a three point titration. The three points are 0.5:1, 1:1, 2:1 (protein:"naked" DNA/mononucleosome) also containing RB, DB and sterile water. The native PAGE gel showed no shift in either "naked" DNA or mononucleosome in any INI1 Δ1-62/Δ186-385 combined INI1 Δ105-385 lanes (Figure 18, 19).

Titration of Δ1-105/186-385, and Δ105-385 combined and Δ1-63/Δ186-385 and Δ1-186 combined with "Naked" DNA

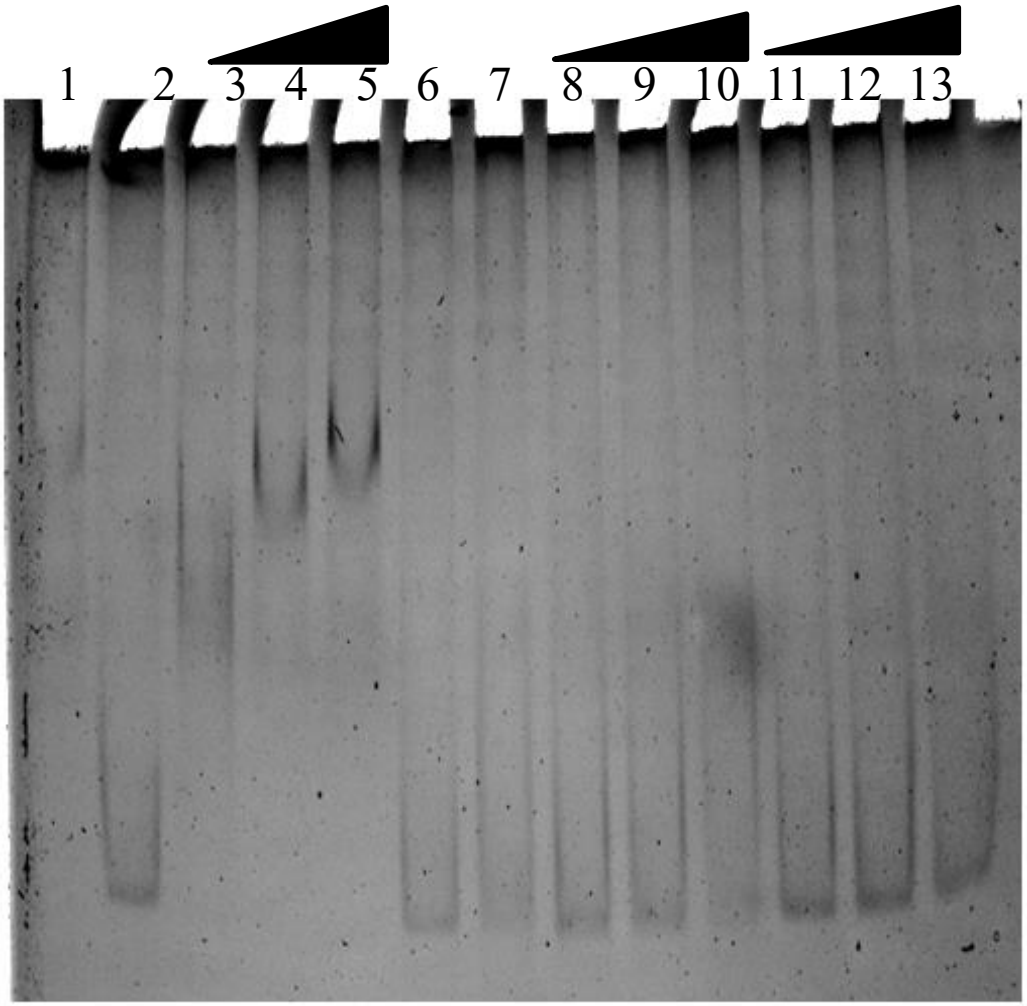

Fig18: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-5: Δ186-385. Lane6: Δ105-385. Lane7: Δ1-105/Δ186-385. Lane 8-10: Δ105-385 and Δ1-105/Δ186-385. Lane 11-13: Δ1-63/Δ186-385 and Δ1-186. Ratio: protein:"Naked" DNA: 1) 2:1 (300 nM:150 nM) 2)0:1 (0 nM:150 nM) 3).5:1 (75 nM:150 nM) 4)1:1 (150 nM:150 nM) 5)2:1 (300 nM:150 nM) 6)2:1 (300 nM:150 nM) 7)2:1 (300 nM:150 nM) 8)0.5:1 (75 nM:150 nM) 9)1:1 (150 nM:150 nM) 10)2:1 (300 nM:150 nM) 11)0.5:1 (75 nM:150 nM) 12)1:1 (150 nM:150 nM) 13)2:1 (300 nM:150 nM) Representative gel of n=2

Titration of Δ1-105/186-385, and Δ105-385 combined and Δ1-63/Δ186-385 and Δ1-186 combined with Mononucleosome

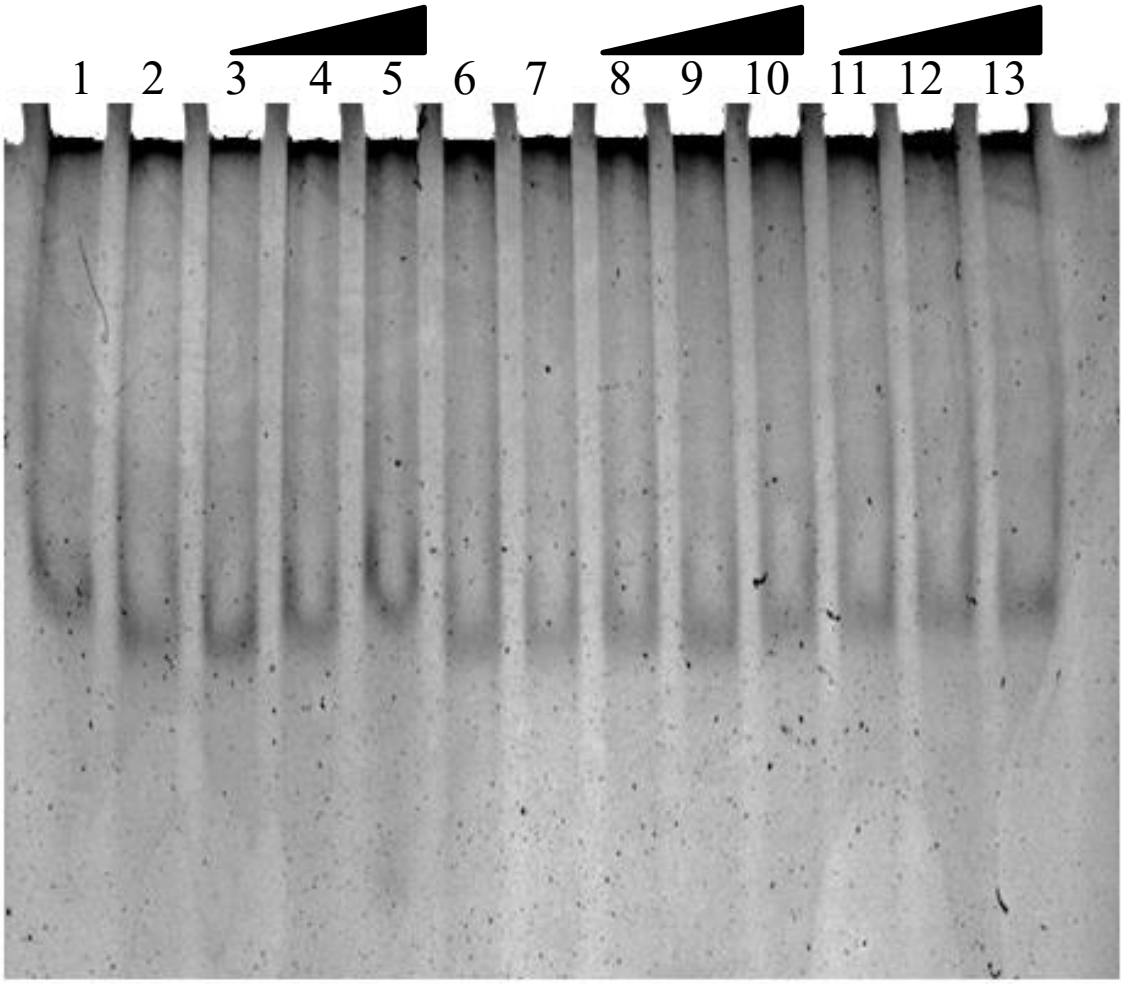

Fig19: Samples were incubated for 25 min. prior to loading on a 6% native PAGE EMSA. Run 85 min in cold. Lane 1: full length INI1. Lane 2: no protein. Lane 3-5: Δ186-385. Lane6: Δ105-385. Lane7: Δ1-105/Δ186-385. Lane 8-10: Δ105-385 and Δ1-105/Δ186-385. Lane 11-13: Δ1-63/Δ186-385 and Δ1-186. Ratio: protein:mononucleosome: 1) 2:1 (300 nM:150 nM) 2)0:1 (0 nM:150 nM) 3)0.5:1 (75 nM:150 nM) 4)1:1 (150 nM:150 nM) 5)2:1 (300 nM:150 nM) 6)2:1 (300 nM:150 nM) 7)2:1 (300 nM:150 nM) 8).5:1 (75 nM:150 nM) 9)1:1 (150 nM:150 nM) 10)2:1 (300 nM:150 nM) 11).5:1 (75 nM:150 nM) 12)1:1 (150 nM:150 nM) 13)2:1 (300 nM:150 nM)
Representative gel of n=2

CHAPTER IV

Discussion

INI1's role within SWI/SNF and several cellular processes (including DNA regulation/repair) has shown a need to characterize and understand its DNA binding abilities and properties. Understanding this function and identify the region will help in determining INI1's role in these purported processes. The decryption of INI1's DNA binding profile has the potential of giving new insight into its role in the diseases associated with INI1 malfunction.

Protein Purification

The INI1's proteins expressed were purified once using affinity chromatography. Due to the nature of protein purification there were common contaminating *E. coli* proteins. The purifications of INI1 Δ186-385, and INI1 Δ1-186 are shown as examples (Figure 3). The implication is that while samples contained some contamination these contaminating protein bands do not interfere with the binding activity of INI1. This is indicated by the binding profiles of the two INI1 truncations

INI1 Binding Ability

Our lab, along with others shows that full length INI1 binds "naked" DNA; additionally in a similar region, that corresponds to INI1 Δ186-385. Our lab shows that both full length INI1 and INI1 Δ186-385 have differential migration pattern in an EMSA when compared to "naked" DNA with no protein (Figure 4). It is also shown by ours and other labs that the region INI1 Δ1-186 does not demonstrate differential migration in an EMSA when compared to "naked" DNA with no protein (Figure 5). The accepted structure of INI1 is that the region of INI1 Δ1-186 is used in protein-protein interactions [3]. This inability to binding would be consistent with the known activates of this region of INI1. Thus our study provides further evidence that the DBD of INI1 in contained within the INI1 Δ186-385 region (Figure 20).

The lab shows that INI1 can bind mononucleosomes. INI1 Δ186-385 was also shown to have differential migration in an EMSA when compared to mononucleosome with no protein (Figure 7). However, the INI1 Δ1-186 region was shown to have no differential migration in an EMSA when compared to mononucleosome with no protein (Figure 7). The result suggests that INI1 Δ1-186 doesn't bind to the mononucleosome which gives evidence that suggest INI1 is not interacting with the core histones in a manner similar to known INI1 protein-protein interactions. The ability of INI1 to bind both "naked" DNA and mononucleosome is evidence that INI1 is likely only contacting with one face of the mononucleosome as a result INI1 would also bind most likely to a single face of the "naked" DNA. INI1 Δ186-385 is unlikely to surround the mononucleosome when it binds inferring more evidence that only a single face is contacted. The binding of INI1 to a mononucleosome provides the first evidence that INI1 may be playing a role in the attachment of the SWI/SNF chromatin remodeling complex to the nucleosome.

Given that INI1 has the ability to bind both constructs this provides some insights into how INI1 might interact with DNA. The data suggests that INI1 is most likely only making contact with a single face of the "naked" DNA and mononucleosome. This would then imply that

The Binding of INI1 to “Naked” DNA and Mononucleosome

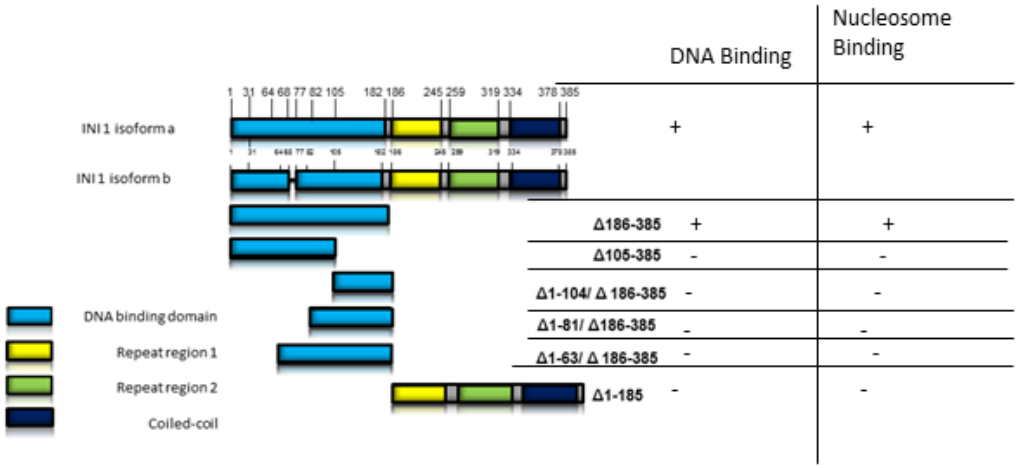

Fig 19

Positive binding: +

Negative binding: -

INI1 does not wrap around the "naked" DNA nor does it wrap around the mononucleosome. The implication is that INI1 is likely to not interact directly with the histone core of the nucleosome. INI1 is likely to be interacting by inserting into a single or multiple major grove(s) as both structures would exist in both "naked" DNA and mononucleosomes topography.

INI1 Truncation and the Binding to DNA and Nucleosome

Our lab shows that INI1 Δ186-385 was able to bind both "naked" DNA and mononucleosomes. The next question to address is how expansive the DBD of INI1 is. The first truncations used INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 both showed no migration difference with "naked" DNA when compared to no protein lane (Figure 8,9). This indicates that the "naked" DNA binding region of INI1 includes part of both fragments. Data also showed that INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 did not migrate differently than the no protein: mononucleosome lane (Figure 10,11). It can be inferred that both truncations do not contain the full region that allows INI1 Δ186-385 to bind to mononucleosome.

Lab results also implicate that the removal of Δ64-385impacts the ability of INI1 to bind DNA. INI truncations Δ1-82/Δ186-385 and Δ1-62/Δ186-385 both fail to show differential migration when compared in an EMSA to "naked" DNA with no protein (Figure 12,14). This leads to the speculation that INI1 Δ64-385 holds an area that seem to contribute to INI1's ability to interact with "naked" DNA. Data also indicated that INI1 Δ1-82/Δ186-385 and Δ1-62/Δ186-385 lacked a differential migration pattern in an EMSA when compared to mononucleosome with no protein (Figure 13,15). This indicates that INI1 Δ64-385 contains an area that contributes to INI1's ability to interact with the mononucleosome.

To understand if differential migration could be restored. The lab added INI1 Δ1-104/Δ186-385 and INI1 Δ105-385 to the same EMSA sample tube. The results show no

differential migration when compared respectively to either "naked" DNA or mononucleosome. This indicates that the INI1 truncation Δ186-385 must be intact to be functional. Given that combined truncations did not bind indicates that the regions of binding spans a region encompassing both INI1 Δ1-104/Δ186-385 and INI1 Δ105-385.

These results along with results from the INI1 Δ1-62/Δ186-385 combined INI1 Δ105-385 native gel indicate that the amount of protein within the EMSA is not accounting for the "recovered" binding ability of the Δ1-104/Δ186-385 and INI1 Δ105-385 combined sample. Additional evidence that protein amount does not affect our EMSA results is the comparison of the Δ1-104/Δ186-385 and INI1 Δ105-385 at 2:1 and 8.67:1 (protein: "naked" DNA). The lack of a differential shift indicates that protein amount is not contributing to the binding ability of INI1 (fig16, 17, 18). This provides further evidence that the DBD for INI1 within an area over lapping INI1 truncations Δ1-104/Δ186-385 and INI1 Δ105-385. Additionally these studies indicate the putative importance of the Δ64-385 region to INI1's ability to bind.

EMSA Ratios

EMSA does not provide enough evidence for stoichiometry determination by itself. Thus the ratios within the EMSAs do not represent the stoichiometry of INI1 binding.

Non-Specific Binding

The duality of INI1 binding also provides evidence that INI1 is binding non-specifically to the DNA in both constructs. Non-specific binding is most likely because of a lack of similar specificity binding sites between "naked" DNA and mononucleosomes that would be needed for the protein attachment. Additionally, the hydrogen bonds that enable non-specific binding do not

have to be the same binding partners in order to provide stability with the different bound complexes.

Future Direction

Further investigation is needed in determining the minimal binding region of INI1. Additionally, investigation is needed in how INI1's incorporation into the SWI/SNF complex impacts binding to "naked" DNA and mononucleosomes. Post translational modifications have been implicated with INI1's putative DNA binding region. Understanding how and when these modifications occur is important in elucidating the vivo binding characteristics of INI1 to "naked" DNA and mononucleosomes. The ultimate goal would be to crystallize the bond INI1 to either "naked" DNA or mononucleosome, which would allow better understanding of the mechanics of binding. Additional, studies are needed into how disruptions in INI1 and INI1's DNA bind impact SWI/SNF function as well as, INI1's role in the development of AT/RT cancer.

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VITA

Dustin James Steele

Candidate for the Degree of

Master of Science

Thesis: CHARACTIZATION OF INTERGRASE INTERACTOR 1 BINDING

Major Field: Biochemistry and Molecular Biology

Biographical:

Education:

Completed the requirements for the Master of Science in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in July, 2016.

Completed the requirements for the Bachelor of Science in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in 2013.